Inhibition of cyclin-dependent kinase activity exacerbates H202-induced DNA damage in Kindler syndrome keratinocytes

Concise Communication:

Inhibition of cyclin-dependent kinase activity exacerbates H_20_2-induced DNA damage in Kindler syndrome keratinocytes

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Running title: Cyclin-dependent kinases and Kindler syndrome

Abstract

Kindler Syndrome (KS) is an autosomal recessive skin disorder characterized by skin blistering and photosensitivity. KS is caused by loss of function mutations in *FERMT1*, which encodes Kindlin-1. Kindlin-1 is a FERM domain containing adaptor protein that is found predominantly at cell-extracellular matrix adhesions where it binds to integrin β subunits and is required for efficient integrin activation. Using keratinocytes derived from a patient with KS, into which wild type Kindlin-1 (Kin1WT) has been expressed, we show that Kindlin-1 binds to cyclin-dependent kinase (CDK)1 and CDK2. CDK1 and CDK2 are key regulators of cell cycle progression, however, cell cycle analysis showed only small differences between the KS and KS-Kin1WT keratinocytes. In contrast, G2/M cell cycle arrest in response to oxidative stress induced by hydrogen peroxide (H_2O_2) was enhanced in KS keratinocytes but not KS-Kin1WT cells, following inhibition of CDK activity. Furthermore, KS keratinocytes were more sensitive to DNA damage in response to H_2O_2 and this was exacerbated by treatment with the CDK inhibitor roscovitine. Thus, in Kindlin-1 deficient keratinocytes, CDK activity can further regulate oxidative damage induced cell cycle arrest and DNA damage. This provides further insight into the key pathways that control sensitivity to oxidative stress in KS patients.

KEYWORDS

CDK1, CDK2, Kindlin-1, cell cycle, DNA damage

1 BACKGROUND

Kindlin-1 is one of three closely related proteins (Kindlin-1, -2 and -3) consisting primarily of a four.1, ezrin, radixin and moesin (FERM) domain split by a pleckstrin homology domain.[1] Kindlins play a key role in connecting dynamic actin regulation with membrane rearrangements at the leading edge of motile cells through their ability to regulate integrin activation.[2, 3] Loss of function mutations in the *FERMT1* gene, which encodes Kindlin-1 leads to an autosomal skin disorder called KS.[4, 5] Patients with KS show photosensitivity, skin blistering in sun-exposed areas and poikiloderma.[5-7] Disruption of integrin-dependent functions of Kindlin-1 have been implicated in a number of pathologies associated with KS but there remain many questions about how loss of Kindlin-1 regulates the behaviour of keratinocytes and the aetiology of KS.

2 QUESTIONS ADDRESSED

Previously we have shown that Kindlin-1 can translocate to centrosomes, where it regulates mitotic spindle formation and can regulate cell division in the skin of mice.[8, 9] We therefore asked whether Kindlin-1 binds the cell cycle regulators CDK1 and CDK2. Furthermore, we addressed whether the increased sensitivity of KS keratinocytes to oxidative damage was regulated by inhibition of CDK activity.

3 EXPERIMENTAL DESIGN

Immortalized human keratinocytes from a patient harbouring a *FERMT1* mutation (c.676insC/c.676insC) were used in this study.[10] To address Kindlin-1 dependent effects wild-type Kindlin1-mCherry was expressed in the KS keratinocytes to generate KS-Kin1WT keratinocytes. Detailed protocols are provided in Data S1. Comparison of cell cycle profiles was carried out with the ImageXpress Micro XLS High-content analysis system by grouping

DAPI stained nuclei according to size and brightness of the DAPI signal. RFP-trap pull downs combined with western blotting was used to assess binding of Kindlin-1 to CDK1 and CDK2. To induce oxidative stress cells were treated with H_2O_2 and phosphorylation of histone H2AX, used as a marker of DNA damage, was analyzed by immunofluorescence. To assess the role of CDKs in response to H_2O_2, the pan-CDK inhibitor roscovitine was used.

3 RESULTS

3.1 Kindlin-1 binds CDKs

As we had previously identified a role for Kindlin-1 in mitotic progression[8, 9] we looked at the interaction between Kindlin-1 and CDKs. RFP-trap agarose beads were used to pull down RFP-Kindlin-1 in KS-Kin1WT keratinocytes using KS keratinocytes as a control. Western blotting showed that both CDK1 and the structurally similar CDK2 associated with Kindlin-1 (Figure 1A). Using recombinant CDK1 and CDK2 we were able to show a direct interaction with Kindlin-1 (Figure S1A). To confirm binding of Kindlin-1 to CDK1/2 we used a mouse squamous cell carcinoma model in which Kindlin-1 had been genetically deleted (SCC-Kin-1-/-) as detailed in Data S1.[17] Immunoprecipitation of CDK1 or CDK2 from SCC-Kin-1-/- cells and SCC-Kin-1WT cells in which Kindlin-1 had been re-expressed, showed that Kindlin-1 associated with both CDK1 and CDK2 (Figure S1A). This was more evident for CDK2 which likely reflects the greater expression of CDK2 in the immunoprecipitates (Figure S1B).

We then looked at the expression of both CDK1 and CDK2 and other cell cycle associated proteins in the KS and KS-Kin1WT keratinocytes. There were no differences in the levels of CDK1 or CDK2 in the two cell lines with the most striking difference being the phosphorylation of CDK1 on tyrosine (Tyr) 15, which was significantly lower in the KS keratinocytes (Figure 1B,C).

3.2 Effect of Kindlin-1 on cell cycle progression

Dephosphorylation on Tyr15 is the critical regulatory step in activating CDK1[11] and to determine whether this change in phosphorylation was associated with changes in cell cycle progression, we analysed the cell cycle in the keratinocytes. In KS keratinocytes there was a small decrease in the percentage of cells in G1 compared to the KS-Kin1WT cells, which was accompanied by an increase in G2/M, both of which were not statistically significant (Figure 1D-F). Furthermore, there was no difference in the proliferation of the KS and KS-Kin1WT keratinocytes (Figure 1G).

As the effects on the cell cycle were small we asked whether cell cycle arrest in response to DNA damage was altered and whether this was dependent on CDK activity using the CDK inhibitor roscovitine. Roscovitine is a pan-CDK inhibitor, which inhibits CDK1, CDK2 and CDK5.[12, 13] H_2O_2 induces cell cycle arrest, with sublethal doses leading to G2/M cell cycle arrest.[14, 15] Treatment with a low concentration of H_2O_2 resulted in a small decrease in G1 with an accompanying increase in G2/M in both KS and KS-Kin1WT keratinocytes (Figure 2A). Treatment with both H_2O_2 and roscovitine however, led to a further decrease in G1 and a concomitant increase in G2/M in KS keratinocytes, which was not seen in the KS-KinWT keratinocytes, resulting in a significant difference in cell cycle arrest between the KS and KS-Kin1WT cells (Figure 2A-C). Treatment with roscovitine reduced phosphorylation of retinoblastoma protein (Rb), demonstrating inhibition of CDK activity (Figure 2D). Thus, in the absence of Kindlin-1, cells have an increased dependence on CDK activity for stress-induced cell cycle arrest.

3.4 Kindlin-1 loss and CDK inhibition synergize to induce DNA damage

Another important function of CDKs is to regulate the cellular response to DNA damage.[16] We know from previous studies that Kindlin-1 deficient cells are more sensitive to oxidative stress and that they exhibit more DNA damage after oxidative stress.[17] To gain further insight into the biological significance of CDKs in this Kindlin-1-dependent response, we analyzed DNA damage following treatment with H_2O_2 in the presence or absence of roscovitine. Treatment with H_2O_2 led to increased accumulation of nuclear phosphorylated H2AX in KS but not KS-Kin1WT cells (Figure 2E, F). Treatment with both roscovitine and H_2O_2 led to a further increase in DNA damage in the Kindlin-1 deficient keratinocytes compared to the KS-Kin1WT cells (Figure 2E-G). Thus, the increased sensitivity of the Kindlin-1 deficient keratinocytes to DNA damage is exacerbated following inhibition of CDK activity, indicating that in the absence of Kindlin-1 CDK activity can alter the DNA damage response. Similar results were seen in the Kindlin-1 deficient SCC cells, with roscovitine treatment leading to increased levels of DNA damage induced by H_20_2 in the SCC-Kin-1-/- cells compared to the SCC-Kin-1WT (Figure S1B,C).

4 CONCLUSIONS

Kindlin-1 is important for integrin activation: integrins are transmembrane receptors that link the extracellular matrix to the actin cytoskeleton.[18, 19] Integrins can cooperate with growth factor receptors to regulate cell cycle progression and proliferation[20] by cooperatively inducing PI3K/AKT, MEK/ERK and small GTPase signalling.[19, 21] However, the proximal integrin downstream signals controlling cell cycle progression are poorly understood. Interestingly, ILK, another focal adhesion protein that has been identified as a binding partner of Kindlin-1, functions downstream of integrins and may be a critical regulator of integrin-mediated cell cycle progression.[22] This is the first report of an adhesion linked protein binding

to CDKs although CDK1 has been shown to phosphorylates a number of proteins (including Kindlin-2) that are involved in regulation of adhesion complexes and the actin cytoskeleton, indicating a role for CDK1 cell cycle-dependent regulation of adhesion [23, 24].

The cell cycle profile of Kindlin-1 deficient keratinocytes did not differ significantly from that of the Kindlin-1 expressing cells. In addition, Kindlin-1 deficiency did not alter keratinocyte proliferation indicating that Kindlin-1 is not required for cell cycle progression in this model. Similar results were seen in the SCC model used in this study where loss of Kindlin-1 did not impact on proliferation in 2D culture (V.G.B, manuscript in preparation). This is in contrast to our previous report in mouse skin where Kindlin-1 deficiency resulted in a reduction in proliferation.[8] In addition, previous studies have reported proliferation defects in Kindlin-1 deficient keratinocytes when compared to normal human keratinocytes.[25, 26] This indicates that there are context dependent differences in the requirement for Kindlin-1 in cell cycle progression and proliferation and that other integrin proximal signals may be able to compensate for loss of Kindlin-1.

CDKs also play an important role in controlling the response to DNA damage and we show that roscovitine, a pan-CDK inhibitor, can exacerbate both the cell cycle arrest and DNA damage induced by H_2O_2 in KS keratinocytes. This has previously been reported for roscovitine in the context of DNA damage induced by chemotherapeutic agents.[27, 28] CDK1 is involved in the DNA double-strand break homologous recombination repair pathway. Inhibition of CDK activity by roscovitine arrests cells in G2/M and primes them for DNA damage which is associated with reduced recruitment of homologous recombination proteins.[27] Both CDK1 and PLK1, which we have previously identified as a Kindlin-1 binding partner[9], are involved in the regulation of DNA double strand break repair by non-homologous end joining (NHEJ) through phosphorylation of 53BP1.[29] This raises the intriguing possibility that Kindin-1 may play an adaptor function in pathways that regulate DNA damage repair.

Keratinocytes from KS patients are more prone to oxidative stress[30] and have increased sensitivity to UV irradiation.[17, 31] A number of Kindlin-1 dependent signaling pathways linked to increased sensitivity in the KS keratinocytes have been identified[17, 31] but here we show that Kindlin-1 binding partners also play an important role. Photosensitivity manifests early in life in KS patients and is a distressing clinical feature of the disease. Understanding the underlying mechanisms that control DNA damage repair pathways in the absence of Kindlin-1 will help identify potential approaches to alleviate the photosensitivity in KS patients.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTIONS

H.E. and J.C. carried out the experiments and data analysis. V.G.B. and H.E. designed the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

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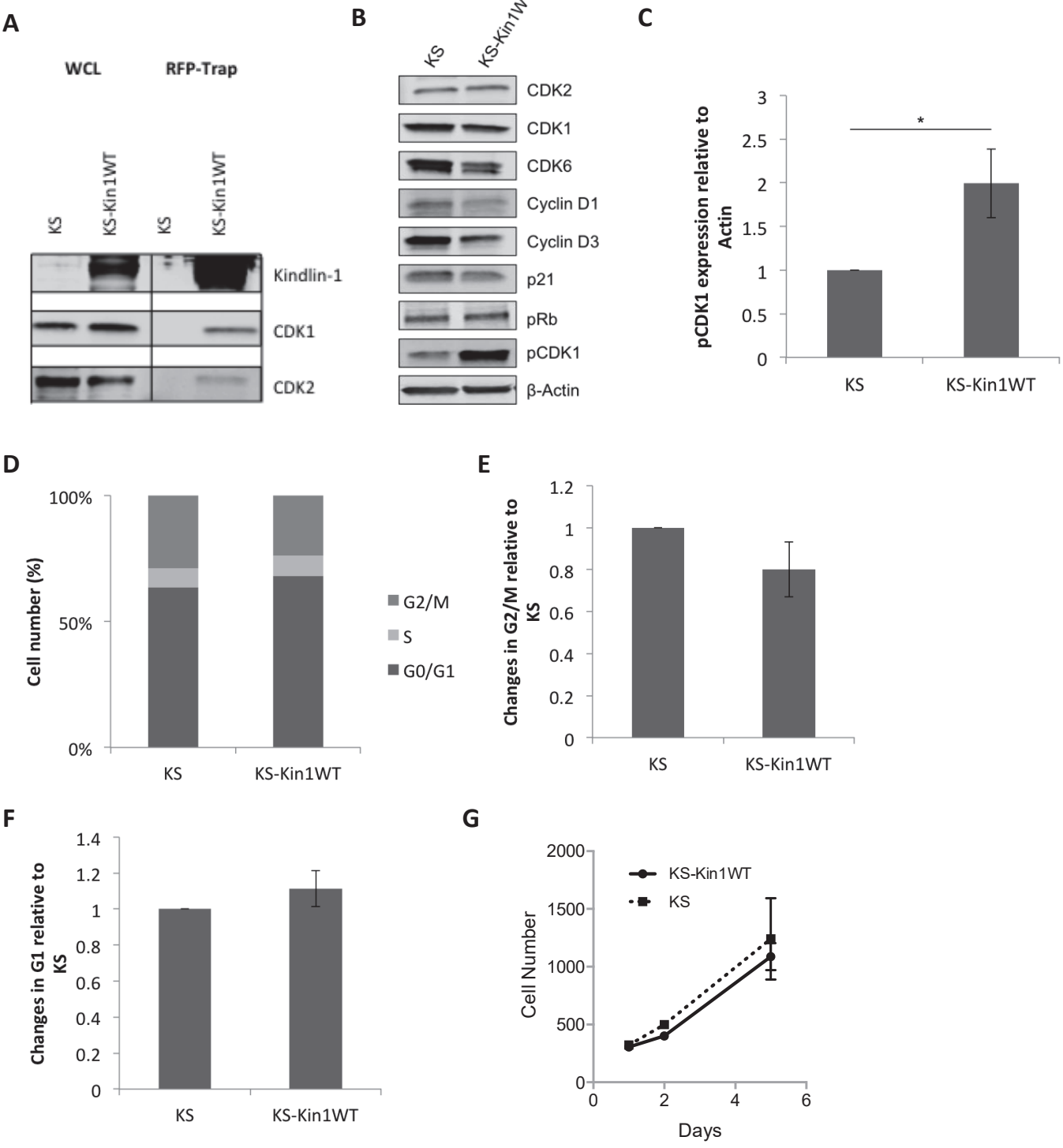
FIGURE LEGENDS

FIGURE 1 Kindlin-1 binds CDKs but does not alter cell cycle progression. A, Western blot analysis of RFP-trap immunoprecipitates and whole cell lysates (WCL) in KS and KS-Kin1WT keratinocytes. B, Western blot analysis of cell cycle regulators in KS and KS-Kin1WT keratinocytes. C, Quantification of pCDK1 levels relative to actin. Results represent the quantification and s.e.m. of 3 independent experiments. *$p<0.05$, statistically significant difference after one-sample student *t*-test. D, Cell cycle profiles of KS and KS-Kin1WT keratinocytes. E, F Relative changes in G2/M (E) and G1 (F) in KS-Kin1WT compared to KS keratinocytes. Results represent the mean and s.e.m. of 4 independent experiments. *$p<0.05$, statistically significant difference after one-sample student *t*-test. G, Quantification of KS and KS-Kin1WT cell number over time.

FIGURE 2 Loss of Kindlin-1 and inhibition of CDKs sensitize cells to cell cycle arrest and oxidative damage in response to hydrogen peroxide. A, Cell cycle profiles of KS and KS-Kin1WT keratinocytes treated with roscovitine and 250 μM H_2O_2. B, C Relative change of G1 (B) and G2/M (C) between cells treated with both roscovitine and H_2O_2 to the untreated control. Data was further normalized to KS. Results represent the mean and s.e.m. of 4 independent experiments. *$p<0.05$, **$p<0.01$, statistically significant difference after paired two-sample *t*-test. D, Western blot analysis of pRb in KS and KS-Kin1WT keratinocytes treated with roscovitine. Actin was used as a loading control. E, F DNA damage measured by pH2AX immunofluorescence staining in KS and KS-Kin1WT keratinocytes treated with roscovitine and 250 μM H_2O_2. Quantification of nuclear pH2AX fluorescence intensity normalized to nuclear DAPI (E) and representative images of pH2AX (green) and DAPI (blue) to visualize nuclei (F). G, Increase in DNA damage normalized to Kindlin-1 expressing cells and H_2O_2

treatment. Results represent the mean and s.e.m. of at least 3 independent experiments. *p<0.05, statistically significant difference from control value after one-sample student *t*-test.

Figure 1


A
WCL
RFP-Trap
KS
KS-Kin1WT
KS
KS-Kin1WT
Kindlin-1
CDK1
CDK2
B
KS
KS-Kin1WT
CDK2
CDK1
CDK6
Cyclin D1
Cyclin D3
p21
pRb
pCDK1
β-Actin
C
pCDK1 expression relative to Actin
3
2.5
2
1.5
1
0.5
0
*
KS
KS-Kin1WT
D
Cell number (%)
100%
50%
0%
G2/M
S
G0/G1
KS
KS-Kin1WT
E
Changes in G2/M relative to KS
1.2
1
0.8
0.6
0.4
0.2
0
KS
KS-Kin1WT
F
Changes in G1 relative to KS
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KS
KS-Kin1WT
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Cell Number
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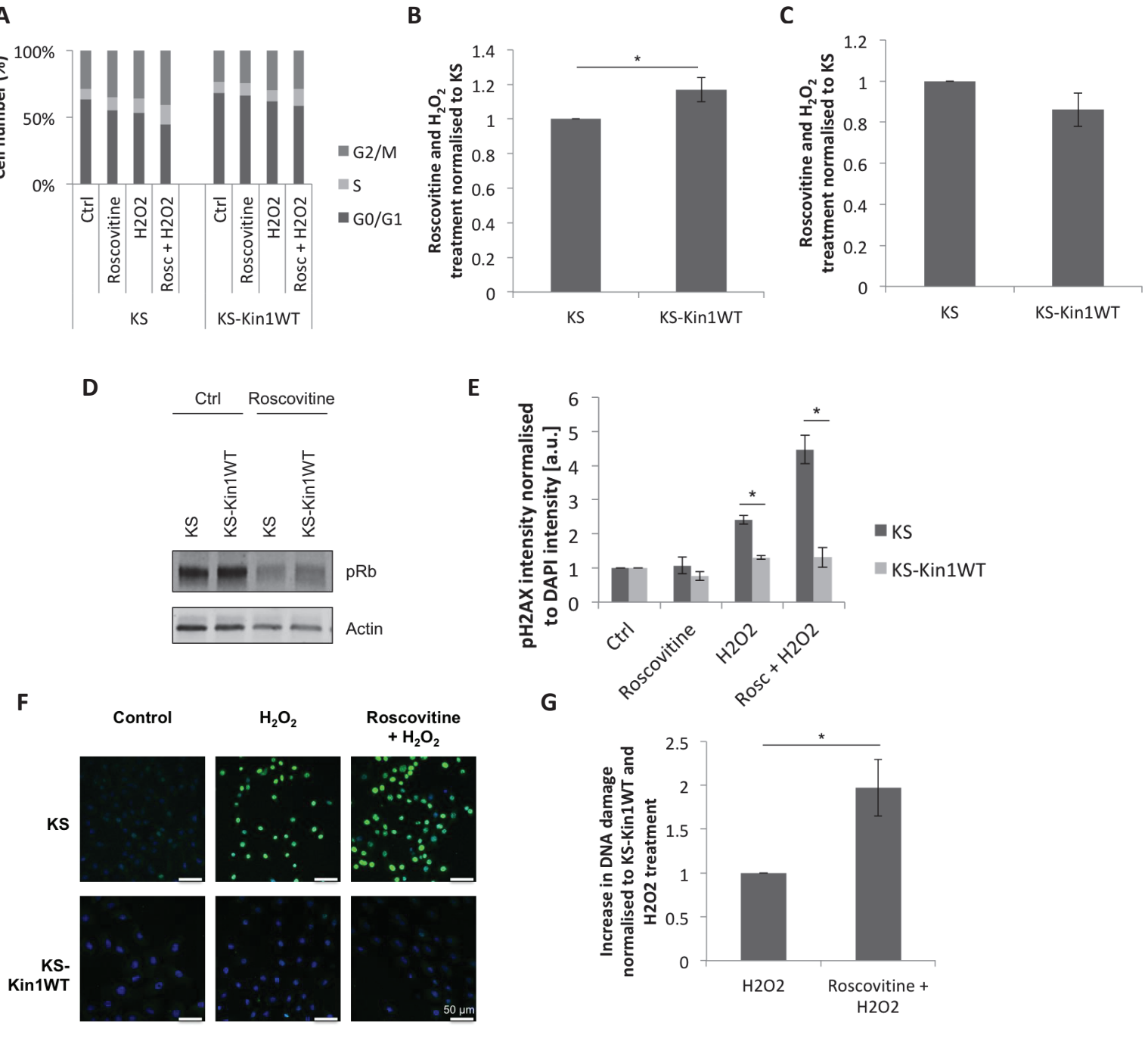
1000
500
0
0
2
4
6
Days
KS-Kin1WT
KS

Figure 2


A
100%
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Cell number (%)
G2/M
S
G0/G1
Ctrl
Roscovitine
H2O2
Rosc + H2O2
KS
KS-Kin1WT
B
Roscovitine and H2O2 treatment normalised to KS
*
KS
KS-Kin1WT
C
Roscovitine and H2O2 treatment normalised to KS
KS
KS-Kin1WT
D
Ctrl
Roscovitine
KS
KS-Kin1WT
pRb
Actin
E
pH2AX intensity normalised to DAPI intensity [a.u.]
KS
KS-Kin1WT
Ctrl
Roscovitine
H2O2
Rosc + H2O2
F
Control
H2O2
Roscovitine + H2O2
KS
KS-Kin1WT
50 µm
G
Increase in DNA damage normalised to KS-Kin1WT and H2O2 treatment
H2O2
Roscovitine + H2O2

Data S1

1.1 Cell culture

Kindlin-1 was sub-cloned into pHR9SIN-SEW lentiviral expression vector containing an N-terminal mCherry tag. Lentiviral DNA was transfected with the Δ8.91 packaging plasmid and pMD2.G envelope plasmid into HEK293 cells for lentiviral particle production. Virus was harvested and used to infect target keratinocytes. Expressing cells were further selected for expression using FACS and maintained for further passages under identical conditions to non-infected cells (Zhang et al., 2016). Keratinocytes were cultivated in KSF-M (Thermo Fisher) at 37°C and 5% CO_2. For the proliferation assay, 250 cells were counted and plated in 6-well plates. Cells were trypsinized and counted each day for up to 7 days. Generation of SCC-Kin1-/- and SCC-Kin1WT cells was previously described (Emmert et al., 2017). SCCs were induced in K14CreER^{T2} Kin1$^{flox/flox}$ mice using a two-stage chemical skin carcinogenesis protocol as previously described (McLean et al., 2004). Following surgical excision of carcinomas, small tissue pieces were adhered to tissue culture plates and cells allowed to grow out in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% foetal bovine serum (FBS). To induce Kindlin-1 deletion SCC cells were cultured in the presence of 15 μM 4-hydroxytamoxifen (4OHT) for 24 hours. Cells were then trypsinized and single cells seeded into each well of a 96-well plate and allowed to establish colonies. Resulting colonies were screened for genetic deletion of *Fermt1* (SCC-Kin1-/-). Wild-type Kindlin-1 (SCC-Kin1WT) was cloned into pWZL-Hygro retroviral vector and introduced into the SCC-Kin1-/- cells or the empty vector and stable pools selected in 0.25 mg/ml hygromycin. SCC cells were cultivated at 37°C and 5% CO_2 in DMEM with 10% FBS. Cells were used at passages 3–20.

1.2 Immunoprecipitation and western blotting

Sub-confluent cells were washed twice in ice-cold PBS and lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (Roche, 04906845001 and 04693124001, respectively) at 4°C. For RFP-trap pull downs 500 μg lysate was incubated with RFP-trap agarose beads (Chromotek) at room temperature for 45 minutes, washed three times in lysis buffer and three times in PBS. For CDK1 and CDK2 immunoprecipitates 500 μg lysate was incubated with either IgG (Cell Signaling Technology #2729), CDK1 (Cell Signaling Technology #9116) or CDK2 (Cell Signaling Technology #2546) antibodies in PBS containing 0.1% Triton-X 100 (PBST) for 30 minutes at room temperature. Samples were washed three times in lysis buffer and three times in PBS. Immunoprecipitates, RFP-trap pull downs or whole cell lysates were separated according to size using SDS–PAGE, transferred onto nitrocellulose membrane, and blocked for 1 hour in TBS containing 0.05% Tween-20 (TBST) and 5% BSA. Membranes were incubated over night at 4°C and for 45 minutes at room temperature for primary and secondary antibodies, respectively. All washes and antibody incubations were carried out in TBST and TBST plus 5% BSA. Antibodies used were: Actin (Cell Signaling Technology #3700), Kindlin-1 (Abcam #ab68041), CDK1 (Cell Signaling Technology #9116), pCDK1 (Cell Signaling Technology #9111), CDK2 (Cell Signaling Technology #2546), pRb (Cell Signaling Technology #9307), CDK6, CyclinD1, CyclinD3, p21 (Cell Signaling Technology regulation kit #9932). Primary antibodies were used at 1:1000 and dye secondary antibodies were used as directed by the manufacturer (Licor).

1.3 Recombinant protein pull-downs

Kindlin-1 (Origene TP317593) was incubated with 20 µl HIS-Select Nickel Affinity Gel (Sigma H0537) alone or in the presence of 200 ng recombinant HIS-tagged CDK1 (Abbexa abx066201) or 200 ng recombinant HIS -tagged CDK2 (Abbexa abx066204) in 25 mM HEPES pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA with protease and phosphatase inhibitors (Roche 5892970001 and 4906845001) for 1 hour at 4°C with rotation. Beads were washed three times with the above buffer and twice in PBS. 10 µl Laemelli buffer was added to the beads and boiled for 5 minutes at 95°C before performing western blotting as described above with anti-Kindlin-1 antibody.

1.4 Induction of oxidative stress

To induce oxidative stress cells were treated with hydrogen peroxide (H_2O_2) (Merck, #31642). For H_2O_2 treatment media was replaced with freshly prepared media containing 250 µM H_2O_2. In control cells the media was switched to fresh media without H_2O_2. In experiments where roscovitine was used, roscovitine was added at a final concentration of 20 µM for 16 hours (Cell Signaling Technology #9885) prior to H_2O_2 treatment.

1.5 H2AX Assay

Cells grown on coverslips were fixed with Fix-buffer (100 mM PIPES, 3.7% paraformaldehyde, 0.2% Triton X-100, 1 mM $MgCl_2$, and 10 mM EGTA, pH 7.4) for 10 minutes and washed three times in Wash buffer (TBS plus 0.1% Triton X-100). The incubation with primary antibodies was at 4°C overnight in Wash buffer plus 2% BSA. Cover slips were washed twice in Wash buffer and incubated with the secondary antibodies for 90 minutes at room temperature in Wash buffer plus 2% BSA. Cover slips were mounted with Vectashield

Mounting Media containing DAPI (Vector Laboratories). Images were captured using an FV-1000 Olympus confocal microscope using a 40× objective. The primary pH2AX antibody was used 1:200 (Cell Signaling Technology #9718) and the dye (Alexa Fluor)-conjugated secondary antibody was used at 1:400. For quantification cell nuclei were manually selected in ImageJ to create a mask. A mask was then used to measure fluorescence intensity of both the DAPI and the pH2AX channel. pH2AX intensity was normalized to the DAPI signal for each image. For each biological replicate at least 3 images and a total of at least 30 cells each per condition were taken.

1.6 Cell cycle analysis

For the cell cycle analysis 5000 keratinocytes were seeded in 96 well plates. Treatment with inhibitors and H_2O_2 was conducted as described above. Plates were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then stained with DAPI. Plates were imaged using the ImageXpress Micro XLS High-content analysis system analysing the DAPI signal. Analysis of the cell cycle profile was carried out using the inbuilt software by grouping cell nuclei according to size and brightness of the DAPI signal.

1.7 References

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Figure S1

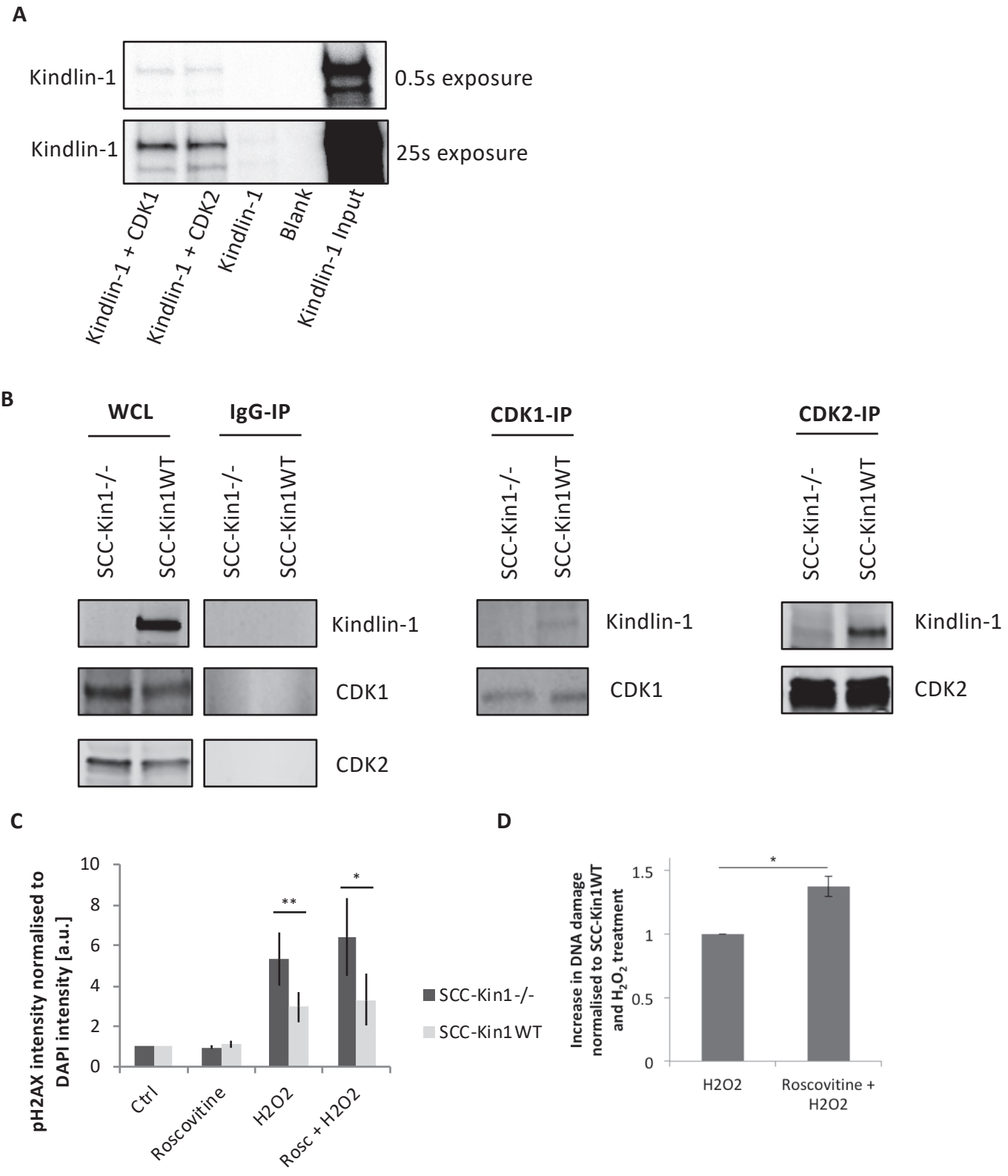


Figure S1: A, Recombinant Kindlin-1 was incubated with either HIS-tagged recombinant CDK1 or CDK2. Following Nickel affinity gel purification western blotting was carried out with a Kindlin-1 antibody showing binding of KIndlin-1 to both CDK1 and CDK2. There was no binding of Kindlin-1 to the Nickel gel in the absence of CDK1/2. Blank represents an unused well and the input is the recombinant Kindlin-1 protein alone. B, Kindlin-1 binds CDK1/2 in SCC cells. Expression of Kindlin-1, CDK1 and CDK2 in whole cell lysates (WCL) of SCC-Kin1-/- and SCC-Kin1WT cells. Kindlin-1 binds to CDK1 and CDK2 immunoprecipitates but not IgG controls in SCC-Kin1WT cells. C, Quantification of nuclear pH2AX fluorescence intensity normalized to nuclear DAPI in SCC cells. D, Increase in DNA damage normalized to SCC-Kin1WT cells and H_2O_2 treatment. Results represent the mean and s.e.m. of at least 3 independent experiments. *p<0.05, **p<0.01 student *t*-test.

May, 21st, 2019

EXD-18-0352.R3 - Inhibition of cyclin-dependent kinase activity exacerbates H202-induced DNA damage in Kindler syndrome keratinocytes

Dear editor,

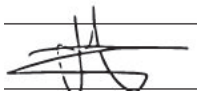
Jayne Culley was added as a co-author, as she has conducted experiments and contributed new data to the revised manuscript.

Thank you.

Kind regards,

Hila Emmert

Valerie Brunton



Jayne Culley